CARBONYL REDUCTASES, POLYNUCLEOTIDES COMPRISING DNA ENCODING THE SAME, METHODS FOR PRODUCING THE SAME, AND METHODS FOR PRODUCING OPTICALLY ACTIVE ALCOHOL UTILIZING THE SAME

FIELD OF THE INVENTION

The present invention relates to novel carbonyl reductases that are dependent on reduced β-nicotinamide adenine dinucleotide phosphate (hereinafter, also referred to as NADPH). The present invention also relates to polynucleotides encoding these enzyme proteins, methods for producing the enzymes, and methods for producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol using the enzymes.

BACKGROUND OF THE INVENTION

(S)-1-(3,4-dimethoxyphenyl)-2-propanol was conventionally produced by reducing 3,4-dimethoxyphenylacetone using microorganisms (see Unexamined Published Japanese Patent Application No. (JP-A) Hei 8-325188 and JP-A Hei 8-89261). However, these methods are not very productive, and only yield the product in a concentration of 1% or less. For this reason, there was a need in the art to establish a simple and highly economical method of obtaining (S)-1-(3,4-dimethoxyphenyl)-2-propanol with a high optical purity and at a high reaction yield.

SUMMARY OF THE INVENTION

An objective of the present invention is to provide methods for efficiently producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol with a high optical purity.

Another objective of the present invention is to provide novel enzymes that reduce 3,4-dimethoxyphenylacetone by utilizing NADPH as a coenzyme to produce (S)-1-(3,4-dimethoxyphenyl)-2-propanol with a high optical purity. A further objective of the present invention is to isolate DNA that encode enzymes comprising the desired property, and to obtain the DNA as recombinant DNA. In addition, a further objective is to provide methods for producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol using this recombinant.

In the search for a simple and highly economical method for

producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol with a high optical purity, the present inventors focused on a method of overexpressing, in a heterologous microorganism, an enzyme that stereo-selectively reduces 3,4-dimethoxyphenylacetone to produce (S)-1-(3,4-dimethoxyphenyl)-2-propanol, and then using the resulting highly active genetically recombinant bacterium to efficiently produce (S)-1-(3,4-dimethoxyphenyl)-2-propanol from 3,4-dimethoxyphenylacetone. The present inventors found that *Torulaspora delbrueckii* had a high reaction yield and high stereoselectivity. They then conducted studies on enzymes in this bacterial strain, which are involved in the reduction of 3,4-dimethoxyphenylacetone . Electrophoresis of a cell-free extract of this bacterial strain showed that an enzyme could be purified to a single electrophoretic band, thus clarifying some of the enzyme's basic properties. As a result, this enzyme was found to be a novel carbonyl reductase that reduces various carbonyls. In addition, the present enzyme reduced 3,4-dimethoxyphenylacetone to produce (S)-1-(3,4-dimethoxyphenyl)-2-propanol with a high optical purity and at a high yield.

Furthermore, the present inventors isolated a DNA encoding the present enzyme, and produced a recombinant bacterium which overexpresses this enzyme, thus completing the present invention. That is, the present invention relates to carbonyl reductases, polynucleotides comprising DNA that encode these enzymes, methods for producing these enzymes, and the use of these enzymes, as set out below.

In the art it is well known that the enzyme 'phenylacetoaldehyde reductase' reduces 3,4-dimethoxyphenylacetone (Eur. J. Biochem., 269, 2394-2402 (2002)). This enzyme reduces ketones in an NADH-dependent manner, and comprises the activity of dehydrogenating secondary alcohols also in an NADH-dependent manner. Thus, it has properties different from the carbonyl reductases of the present invention.

In addition, it is also well known in the art that the enzyme ketoreductase (Eur. J. Biochem., 267, 5493-5501 (2000)), produced by *Zygosaccharomyces rouxii*, can reduce 3,4-methylenedioxyphenylacetone, which has a structure similar to

Zygosaccharomyces rouxii 3,4-dimethoxyphenylacetone. However, there are no reports of its stereoselectivity or activity towards 3,4-dimethoxyphenylacetone. Furthermore, the properties of this enzyme, such as a molecular weight of 42,000 in SDS-PAGE, an optimal pH of 6.6 to 6.8, and an optimal temperature of 37 to 39°C, are different to those of the carbonyl reductases of the present invention.

Furthermore, using the amino acid sequence described in SEQ ID NO: 2, the present inventors performed a SWISS-PROT homology search using a BLAST program. They found proteins homologous to the carbonyl reductases of the present invention. Specifically, genome analysis of *Saccharomyces cerevisiae* resulted in four kinds of predicted ORFs, designated as YGL157w, YGL039w, YDR541c, and YOL151w, respectively. Of these, the functions of proteins encoded by YGL157w, YGL039w, and YDR541c were unknown. YOL151w's activity in reducing various carbonyl compounds had been measured in J. Am. Chem. Soc., 123(8), 1547-1555(2001), but there were no reports of its activity on 3,4-dimethoxyphenylacetone.

Thus, the present invention relates to novel carbonyl reductases, polynucleotides encoding these enzymes, methods for producing these enzymes, methods for producing an optically active alcohol utilizing these enzymes, and uses thereof, as set out below. More specifically, the present invention provides:

[1] a carbonyl reductase comprising the physicochemical properties as shown in (1) and (2),

(1) action

reduces ketones to produce an optically active alcohol, by utilizing reduced β-nicotinamide adenine dinucleotide phosphate as a coenzyme,

(2) substrate specificity

(a) utilizes reduced β-nicotinamide adenine dinucleotide phosphate as a coenzyme in the reduction reaction,

(b) reduces 3,4-dimethoxyphenylacetone to produce (S)-1-(3,4-dimethoxyphenyl)-2-propanol,

(c) comprises the activity of reducing 3,4-dimethoxyphenylacetone, but lacks the activity of oxidizing (S)-1-(3,4-dimethoxyphenyl)-2-propanol;

[2] the carbonyl reductase of [1], which additionally comprises the physicochemical properties of (3) and (4),

(3) optimal pH

pH 5.5 to 6.5,

(4) molecular weight

a molecular weight, determined via sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration, of about 38,000;

[3] the carbonyl reductase of [1], which is produced by a microorganism belonging to the genus *Torulaspora*;

[4] the carbonyl reductase of [3], wherein the microorganism belonging to the genus *Torulaspora* is *Torulaspora delbrueckii*;

[5] a polynucleotide of the following (a) or (b),

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1

(b) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 2;

[6] a polynucleotide encoding a protein comprising the physicochemical properties of (1) and (2) in [1], wherein said polynucleotide is any one of the following (c) to (e),

(c) a polynucleotide encoding a protein comprising an amino acid sequence in which one or more amino acid(s) in the amino acid sequence of SEQ ID NO: 2 has been substituted, deleted, inserted, and/or added ,

(d) a polynucleotide which hybridizes with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 under stringent conditions,

(e) a polynucleotide encoding an amino acid sequence comprising 70% or more homology to the amino acid sequence of SEQ ID NO: 2;

[7] a protein encoded by the polynucleotide of [5] or [6];

[8] a recombinant vector, which comprises the polynucleotide of [5] or [6];

[9] the recombinant vector of [8], which further comprises a dehydrogenase gene for regenerating a coenzyme;

[10] a transformant, which is transformed with the polynucleotide of [5] or [6], or the recombinant vector of [8] or [9];

[11] a carbonyl reducing agent comprising a protein comprising the physiochemical properties of (1) and (2) in [1], and comprising

the function of producing at least 80%ee or more (S)-1-(3,4-dimethoxyphenyl)-2-propanol, wherein said protein is encoded by a polynucleotide according to any one of (a) to (c),

(a) a polynucleotide encoding a protein comprising an amino acid sequence in which one or more amino acid(s) in the amino acid sequence of SEQ ID NO: 17, 21, or 25 has been substituted, deleted, inserted, and/or added,

(b) a polynucleotide which hybridizes with a polynucleotide comprising a nucleotide sequence of SEQ ID NO: 16, 20, or 24 under stringent conditions,

(c) a polynucleotide encoding an amino acid sequence comprising 70% or more homology to the amino acid sequence of SEQ ID NO: 17, 21, or 25;

[12] a method for producing the enzyme of [1], which comprises the step of culturing a microorganism which belongs to genus *Torulaspora* and produces the enzyme of [1];

[13] the method of [12], wherein the microorganism belonging to the genus *Torulaspora* is *Torulaspora delbrueckii*;

[14] a method for producing the carbonyl reducing agent of [9], which comprises the step of culturing a transformant which has been transformed with a recombinant vector that comprises a polynucleotide according to any one of (a) to (e) as follows,

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 16, 20, or 24,

(b) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 17, 21, or 25,

(c) a polynucleotide encoding a protein comprising an amino acid in which one or more amino acid(s) is substituted, deleted, inserted, and/or added to the amino acid sequence of SEQ ID NO: 17, 21, or 25,

(d) a polynucleotide which hybridizes with a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 16, 20, or 24 under stringent conditions,

(e) a polynucleotide encoding an amino acid sequence comprising 70% or more homology to the amino acid sequence of SEQ ID NO: 17, 21, or 25;

[15] a method for producing an optically active alcohol, which

comprises reacting a carbonyl reductase of any one of [1] to [4], the protein of [7], a microorganism producing the enzyme or the protein, the treated microorganism, the transformant of [10], or the carbonyl reducing agent of [11] with a ketone;

[16] a method for producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol, which comprises reacting a carbonyl reductase of any one of [1] to [4], the protein of [7], a microorganism producing the enzyme or the protein, the treated microorganism, the transformant of [10], or the carbonyl reducing agent of [11] with 3,4-dimethoxyphenylacetone.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an SDS-PAGE pattern. Lane 1 indicates a molecular weight marker and lane 2 indicates the enzyme obtained in Example 1.

Fig. 2 is a graph showing the pH dependency of the 3,4-dimethoxyphenylacetone-reducing activity of the enzyme obtained in Example 1. An open circle represents a Britton and Robinson buffer, a triangle represents an acetic acid-sodium acetate buffer, and a filled circle represents a potassium phosphate buffer.

Fig. 3 is a graph showing temperature dependency of the 3,4-dimethoxyphenylacetone-reducing activity of the enzyme obtained in Example 1.

Fig. 4 is a diagram showing the construction of a plasmid (pSE-TDX) into which a part of *Torulaspora delbrueckii*-derived TdCR1 has been introduced.

Fig. 5 is a diagram showing the construction of a plasmid (pUC-TDX) into which a part of *Torulaspora delbrueckii*-derived TdCR1 has been introduced.

Fig. 6 is a diagram showing the construction of a plasmid (pSG-TDX) into which a part of *Torulaspora delbrueckii*-derived TdCR1 has been introduced.

Fig. 7 is a diagram showing the construction of a plasmid (pSE-TDR1) which comprises the full-length TdCR1 gene and can express TdCR1.

Fig. 8 is a diagram showing the construction of a plasmid

(pSG-TDR1) which can coexpress glucose dehydrogenase and TdCR1.

Fig. 9 is a diagram showing the construction of a plasmid (pSE-YGP7) into which a *Saccharomyces cerevisiae*-derived YGL157w gene is introduced. In the plasmid map, P represents a trc promoter, T(rrnB) represents an rrnBT1T2 terminator, amp represents β-lactase gene exhibiting ampicillin resistance, ori represents an origin of replication, rop represents a ROP-protein gene, and laqIq represents a lactose represser.

Fig. 10 is a diagram showing the construction of a plasmid (pSG-YGP7) into which a *Saccharomyces cerevisiae*-derived YGL157w gene and a Bacillus subtilis-derived glucose dehydrogenase gene have been introduced. In the plasmid map, P represents a trc promoter, T(rrnB) represents an rrnBT1T2 terminator, amp represents a β-lactase gene exhibiting ampicillin resistance, ori represents an origin of replication, rop represents a ROP-protein gene, laqIq represents a lactose represser, and BsGlcDH represents a Bacillus subtilis-derived glucose dehydrogenase gene.

Fig. 11 is a diagram showing the construction of a plasmid (pSE-YGD9) into which a *Saccharomyces cerevisiae*-derived YGL039w gene has been introduced. In the plasmid map, P represents a trc promoter, T(rrnB) represents an rrnBT1T2 terminator, amp represents a β-lactase gene exhibiting ampicillin resistance, ori represents an origin of replication, rop represents a ROP-protein gene, and laqIq represents a lactose repressor.

Fig. 12 is a diagram showing the construction of a plasmid (pSG-YGD9) into which a *Saccharomyces cerevisiae*-derived YGL039w gene and a Bacillus subtilis-derived glucose dehydrogenase gene have been introduced. In the plasmid map, P represents a trc promoter, T(rrnB) represents an rrnBT1T2 terminator, amp represents a β-lactase gene exhibiting ampicillin resistance, ori represents an origin of replication, rop represents a ROP-protein gene, laqIq represents a lactose repressor, and BsGlcDH represents a Bacillus subtilis-derived glucose dehydrogenase gene.

Fig. 13 is a diagram showing the construction of a plasmid (pSE-YDR1) into which a *Saccharomyces cerevisiae*-derived YDR541c gene has been introduced. In the plasmid map, P represents a trc promoter,

T(rrnB) represents an rrnBT1T2 terminator, amp represents a β-lactase gene exhibiting ampicillin resistance, ori represents an origin of replication, rop represents a ROP-protein gene, and laqIq represents a lactose repressor.

Fig. 14 is a diagram showing the construction of a plasmid (pSG-YDR1) into which a *Saccharomyces cerevisiae*-derived YDR541c gene and a Bacillus subtilis-derived glucose dehydrogenase gene have been introduced. In the plasmid map, P represents a trc promoter, T(rrnB) represents an rrnBT1T2 terminator, amp represents a β-lactase gene exhibiting ampicillin resistance, ori represents an origin of replication, rop represents a ROP-protein gene, laqIq represents a lactose repressor, and BsClcDH represents a Bacillus subtilis-derived glucose dehydrogenase gene.

DETAILED DESCRIPTION OF THE INVENTION

The carbonyl reductases of the present invention can utilize NADPH as a coenzyme, have no alcohol dehydrogenating activity, and reduce 3,4-dimethoxyphenylacetone by utilizing NADPH as a coenzyme to produce 90%ee or more (S)-1-(3,4-dimethoxyphenyl)-2-propanol.

In the present invention, the activity of reducing 3,4-dimethoxyphenylacetone can be confirmed, for example, as follows:

A method of measuring the activity of reducing 3,4-dimethoxyphenylacetone:

A reaction solution comprising 50 mM potassium phosphate buffer (pH 6.5), 0.2 mM NADPH, 5 mM 3,4-dimethoxyphenylacetone, and an enzyme is reacted at 30°C. Any decrease in absorbance at 340 nm accompanying a decrease in NADPH is measured. 1U is defined as the amount of enzyme which catalyzes a 1 μmol decrease of NADPH in one minute.

A carbonyl reductase comprising the aforementioned physiochemical properties can be purified, for example, from a culture of yeast of the genus *Torulaspora*. In particular, *Torulaspora delbrueckii* is excellent for producing a carbonyl reductase of the present invention. Examples of *Torulaspora delbrueckii* which can be utilized for obtaining a carbonyl reductase of the present invention include IFO 0381 and JCM 5921.

Torulaspora delbrueckii can be cultured in a medium generally used for yeast culture, such as the YM medium. The microorganism is sufficiently grown, and the cells are collected. To obtain a cell-free extract, these cells are then disrupted in a buffer comprising a protease inhibitor and a reducing agent such as 2-mercaptoethanol and phenylmethanesulfonyl fluoride. The enzyme can be purified from the cell-free extract by appropriately combining fractionation utilizing the solubility of the protein (precipitation with an organic solvent and salting out with ammonium sulfate); cation exchange, anion exchange, gel filtration, or hydrophobic chromatography; or affinity chromatography using a chelate, pigment, or antibody, etc. For example, the enzyme can be purified to an electrophoretically single band by hydrophobic chromatography using Phenyl-Sepharose™, anion exchange chromatography using MonoQ, hydrophobic chromatography using Butyl-Sepharose™, adsorption chromatography using hydroxyapatite, etc.

The *Torulaspora delbrueckii*-derived carbonyl reductases of the present invention are proteins comprising the following physiochemical properties of (1) and (2):

(1) action

reduces ketones to produce an optically active alcohol by utilizing NADPH as a coenzyme;

(2) substrate specificity

(a) utilizes NADPH as a coenzyme in the reduction reaction,

(b) reduces 3,4-dimethoxyphenylacetone to produce (S)-1-(3,4-dimethoxyphenyl)-2-propanol,

(c) comprises the activity of reducing 3,4-dimethoxyphenylacetone, but lacks the activity of oxidizing (S)-1-(3,4-dimethoxyphenyl)-2-propanol.

In addition, the carbonyl reductases of the present invention preferably comprise the physicochemical properties of (3) and (4):

(3) optimal pH

pH 5.5 to 6.5,

(4) molecular weight

a molecular weight, determined via SDS-PAGE and gel filtration, of about 38,000.

The present invention relates to polynucleotides that encode carbonyl reductases, and their homologs. In this invention, the term "polynucleotide" refers to a polynucleotide removed from its original environment (e.g., the natural environment if naturally occurring) and thus, altered by the "hand of man" from its natural state. The term therefore covers, for example, (a) a DNA fragment of a naturally occurring genomic DNA molecule free of the coding sequences that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA in the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are nucleic acids present in random, uncharacterized mixtures of different DNA molecules, transfected cells, or cell clones, e.g., as these occur in a DNA library such as a cDNA or genomic DNA library. There is no restriction on the length of the polynucleotide of the present invention, though it preferably comprises at least about 15 nucleotides, more preferably at least about 20, 30, 40, or 50 nucleotides, even more preferably at least about 100, 150, 200, 300, 400, 500, 1000, or 1500 nucleotides. The polynucleotides encoding the carbonyl reductases of the present invention comprise the nucleotide sequence of SEQ ID NO: 1. The nucleotide sequence of SEQ ID NO: 1 encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 2. The protein comprising this amino acid sequence constitutes preferred embodiments of the carbonyl reductases of the present invention.

A homologue of a polynucleotide encoding a carbonyl reductase of the present invention comprises a polynucleotide encoding a protein comprising the above-mentioned physicochemical properties (1) and (2), and comprising the amino acid sequence of SEQ ID NO: 2 in which one or more amino acids are deleted, substituted, inserted, and/or added. Amino acid substitutions may be made at one or more predicted,

preferably nonessential amino acid residues. A "nonessential" amino acid residue is a residue that can be altered from the wild-type sequence of a protein without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. An amino acid is preferably substituted for a different amino acid(s) that allows the properties of the amino acid side-chain to be conserved. Accordingly, a "conservative amino acid substitution" is a replacement in which the amino acid residue is replaced with an amino acid residue having a chemically similar side chain. Groups of amino acid residues having similar side chains have been defined in the art. These groups include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). One skilled in the art can obtain the homolog of the polynucleotide by introducing a substitution, deletion, insertion, and/or addition mutation into the polynucleotide of SEQ ID NO: 1 using standard methods such as site-directed mutagenesis (Nucleic Acid Res. 10, pp. 6487 (1982); Methods in Enzymol. 100, pp. 448 (1983); Molecular Cloning 2nd Ed., Cold Spring Harbor Laboratory Press (1989); and PCR: A Practical Approach, IRL Press, pp. 200 (1991)).

In addition, homologs of the polynucleotide of the present invention include polynucleotides hybridizing under stringent conditions to a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1, and encoding a protein comprising the above-mentioned physicochemical properties (1) and (2). Hybridization may be performed with buffers that permit the formation of a hybridization complex between nucleic acid sequences that contain some mismatches. The phrase "polynucleotide hybridizing under stringent conditions" means a polynucleotide hybridizing to a probe DNA that comprises one or more segments of at least 20 consecutive nucleotides, preferably at least 30 consecutive nucleotides, for example, 40, 60, or 100 consecutive nucleotides, arbitrarily selected from the nucleotide

sequence of SEQ ID NO: 1 using methods such as ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences) under conditions recommended in the attached manual (for example, washing with the primary wash buffer comprising 0.5xSSC at 42°C). Many factors determine the stringency of hybridization, including G+C content of the cDNA, salt concentration, and temperature. For example, stringency may be increased by reducing the concentration of salt or by raising the hybridization temperature. Temperature conditions for hybridization and washing greatly influence stringency and can be adjusted using melting temperature (Tm). Tm varies with the ratio of constitutive nucleotides in the hybridizing base pairs, and with the composition of the hybridization solution (concentrations of salts, formamide and sodium dodecyl sulfate). In solutions used for some membrane-based hybridization, addition of an organic solvent, such as formamide, allows the reaction to occur at a lower temperature. Accordingly, on considering the relevant parameters, one skilled in the art can select appropriate conditions to achieve a suitable stringency based experience or experimentation. More specifically, the expression "stringent conditions" generally refers to 42°C, 2x SSC, and 0.1% SDS; preferably 50°C, 2x SSC, and 0.1% SDS; and more preferably to 65°C, 0.1x SSC, and 0.1% SDS (highly stringent conditions), but is not particularly limited thereto. Polynucleotides isolated under stringent condition as described above are expected to encode polypeptides with higher homology at the amino acid level to the amino acid sequence shown in SEQ ID NO: 2.

Furthermore, the homologs of the polynucleotides of the present invention include a polynucleotide encoding a protein comprising at least 70% homology, preferably at least 80% homology, more preferably 90% or more, and most preferably 95% or more homology to the amino acid sequence of SEQ ID NO: 2. Protein homology searches can be performed, for example, on the Internet, in databases for protein amino acid sequences, such as SWISS-PROT, PIR, and DAD; DNA sequence databases, such as DDBJ, EMBL, and GenBank; databases for deduced amino acid sequences based on DNA sequences; by using the FASTA program, BLAST program, etc.

For the amino acid sequence of SEQ ID NO: 2, a homology search

was performed on DAD using the BLAST program. As a result, among the known proteins, YOL151w (61%) produced by *Saccharomyces cerevisiae*, and ketoreductase (45%) produced by *Zygosaccharomyces rouxii* showed high homology. The expression " or more homology" in the present invention represents a value calculated using, for example, a program of the Lipman-Pearson method (Science, 227, 1435-1441(1985)).

Specifically, a preferred embodiment of the present invention provides a polynucleotide according to any one of (c) to (e), as shown below, that encodes a protein comprising the physiochemical properties (1) and (2), shown above, as well as a protein encoded by the polynucleotide of the present invention.

(c) A polynucleotide encoding a protein comprising an amino acid sequence in which one or more amino acid(s) is substituted, deleted, inserted, and/or added to the amino acid sequence of SEQ ID NO: 2.

(d) A polynucleotide which hybridizes with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 under stringent conditions.

(e) A polynucleotide encoding an amino acid sequence comprising 70% or more homology to the amino acid sequence of SEQ ID NO: 2.

In addition, the BLAST search found predicted open reading frames (ORFs) homologous to the carbonyl reductase of the present invention. However, the functions of these ORFs are unknown. Specifically, genome analysis of *Saccharomyces cerevisiae* resulted in three kinds of predicted ORF, designated as YGL157w, YGL039w, and YDR541c respectively. These predicted amino acid sequences have 57%, 56%, and 61% homology to the carbonyl reductases of the present invention. In order to determine whether these predicted proteins comprise the carbonyl reductase activity of the present invention, primers were synthesized based on DNA sequences registered in DDBJ, and predicted ORFs were cloned from the genomic DNA of *Saccharomyces cerevisiae* using PCR. Each ORF was introduced into an expression vector, *Escherichia coli* (*E. coli*) was transformed to obtain a transformant, and cells were cultured to express each protein. As expected, YGL157w, YGL039w, and YDR541c showed the carbonyl reducing activity. In addition, these homologs reduced 3,4-dimethoxyphenylacetone to produce (S)-1-(3,4-dimethoxyphenyl)-2-propanol with a high optical purity.

Specifically, proteins comprising the amino acid sequence of SEQ ID NO: 17, 21, or 25 represent preferred embodiments of homologs of the carbonyl reductases of the present invention. The ORFs of these proteins were known, but their functions were unknown. The present inventors were the first to suggest that these proteins are carbonyl reductases. These proteins are extremely useful, and can be utilized as carbonyl-reducing agents for producing, for example, optically active alcohols (e.g. (S)-1-(3,4-dimethoxyphenyl)-2-propanol). The present inventors were first to discover the utility of the above proteins as carbonyl-reducing agents.

Therefore, a preferred embodiment of the present invention is a carbonyl-reducing agent, which comprises a protein comprising the physicochemical properties shown in (1) and (2) above, and which is encoded by a polynucleotide of any one of (a) to (e):

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 16, 20, or 24,

(b) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 17, 21, or 25,

(c) a polynucleotide encoding a protein comprising an amino acid sequence in which one or more amino acid(s) is substituted, deleted, inserted, and/or added to the amino acid sequence of SEQ ID NO: 17, 21, or 25,

(d) a polynucleotide which hybridizes with a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 16, 20, or 24 under stringent conditions,

(e) a polynucleotide encoding an amino acid sequence comprising 70% or more homology to the amino acid sequence of SEQ ID NO: 17, 21, or 25.

A further preferred embodiment of the present invention provides a carbonyl-reducing agent, which comprises a protein comprising the physiochemical properties shown in (1) and (2) above, and the function of producing at least 80%ee or more (S)-1-(3,4-dimethoxyphenyl)-2-propanol, wherein the protein is encoded by a polynucleotide of any one of (a) to (c):

(a) a polynucleotide encoding a protein comprising an amino acid sequence in which one or more amino acid(s) is substituted, deleted,

inserted, and/or added to the amino acid sequence of SEQ ID NO: 17, 21, or 25,

(b) a polynucleotide which hybridizes with a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 16, 20, or 24 under stringent conditions,

(c) a polynucleotide encoding an amino acid sequence comprising 70% or more homology to the amino acid sequence of SEQ ID NO: 17, 21, or 25.

The present invention relates to a protein comprising the amino acid sequence of SEQ ID NO: 2. The present invention also comprises a homolog of a protein comprising the amino acid sequence of SEQ ID NO: 2.

"A homolog of the carbonyl reductase" of the present invention means a protein comprising an amino acid sequence in which one or more amino acid(s) is substituted, deleted, inserted, and/or added to the amino acid sequence of SEQ ID NO: 2, where the protein is functionally equivalent to a protein comprising the amino acid sequence of SEQ ID NO: 2. In the present invention, "functionally equivalent to a protein comprising the amino acid sequence of SEQ ID NO: 2" means that the protein has the physiochemical properties shown in the aforementioned (1) to (2). A person skilled in the art can obtain a polynucleotide encoding a homolog of a carbonyl reductase by appropriately introducing a substitution, deletion, insertion, and/or addition mutation in to the DNA of SEQ ID NO: 1 using site-directed mutagenesis (Nucleic Acid Res.10, pp.6487 (1982), Methods in Enzymol.100, pp. 448 (1983), Molecular Cloning 2nd Ed., Cold Spring Harbor Laboratory Press (1989), PCR: A practical Approach IRL Press pp.200 (1991)). By introducing a polynucleotide which encodes a carbonyl reductase homolog into a host, and then expressing it, a homolog of a carbonyl reductase of SEQ ID NO: 2 can be obtained.

The number of amino acids that may be substituted, deleted, inserted, and/or added is not particularly restricted, so long as the protein remains functionally equivalent to a protein comprising the amino acid sequence of SEQ ID NO: 2. Generally, up to about 50 amino acids may be changed, preferably up to about 30 amino acids, more preferably up to about 10 amino acids, and even more preferably

up to about 3 amino acids. Likewise, the site of mutation is not particularly restricted, so long as the mutation does not result in the disruption of the function of the protein.

The carbonyl reductase homologs of the present invention include a protein which comprises at least 70% identity, preferably at least 80% identity, and more preferably 90% or more identity, even more preferably 95% or more homology to the amino acid sequence of SEQ ID NO: 2. Protein homology searches can be performed, for example, on the Internet, using databases for protein amino acid sequences such as SWISS-PROT, PIR, and DAD; DNA sequence databases such as DDBJ, EMBL, or GenBank; or databases for deduced amino acid sequences based on DNA sequences; by using the FASTA program, BLAST program, etc.

A polynucleotide encoding the carbonyl reductase of the present invention may be isolated, for example, using the following procedure:

The DNA of the present invention can be obtained using PCR by designing PCR primers based on the nucleotide sequence of SEQ ID NO: 1, and using chromosomal DNA or a cDNA library of an enzyme-producing strain as a template.

Furthermore, by using the obtained DNA fragment as a probe, a polynucleotide of the present invention can be obtained by colony or plaque hybridization using a cDNA library, or a library obtained by transforming *E. coli* with a phage or plasmid into which restriction enzyme digestion products of the chromosomal DNA of the enzyme-producing strain has been introduced.

A polynucleotide of the present invention can also be obtained by analyzing the nucleotide sequence of the DNA fragment obtained by PCR, using this sequence to design PCR primers that will extend the known DNA outward, digesting the enzyme-producing strain's chromosomal DNA with appropriate restriction enzyme(s), and then using self-ligated circular DNA as a template to perform reverse PCR (Genetics 120, 621-623 (1988)). Alternatively, the Rapid Amplification of cDNA Ends (RACE) method ("Experimental manual for PCR" pp. 25-33, HBJ Press) can be used.

A polynucleotide of the present invention comprises not only genomic DNA and cDNA cloned using the above-mentioned methods, but also chemically synthesized DNA.

By inserting a thus-isolated polynucleotide encoding a carbonyl reductase of the present invention into a known expression vector, a carbonyl reductase expression vector can be provided. That is, the present invention relates to a recombinant vector comprising a polynucleotide of the present invention. A preferred embodiment of the present invention provides a vector comprising a polynucleotide of the present invention. Examples of the vector of the present invention include pSE-TDR1, pSE-YDR1, pSE-YGP7, and pSE-YGD9, in which a gene encoding a carbonyl reductase is introduced in an expressible state in to *E. coli* expression vector pSE420D. In addition, the recombinant vector of the present invention can comprise a coenzyme-regenerating dehydrogenase gene, described below.

Furthermore, by culturing a transformant that has been transformed with this expression vector, a carbonyl reductase of the present invention can be obtained from the transformant.

A specific microorganism transformed to express a carbonyl reductase of the present invention may be any organism, as long as it is transformed with a recombinant vector comprising a polynucleotide encoding a polypeptide comprising a carbonyl reductase, and it can express carbonyl reductase activity. The present invention provides transformants transformed with a polynucleotide of the present invention, or a vector of the present invention.

Non-limiting examples of microorganisms that can be transformed in the present invention are those for which host-vector systems are available, and include the following:

Bacteria such as:

- the genus *Escherichia*
- the genus *Bacillus*
- the genus *Pseudomonas*
- the genus *Serratia*
- the genus *Brevibacterium*
- the genus *Corynebacterium*
- the genus *Streptococcus*
- the genus *Lactobacillus;*

Actinomycetes such as:

- the genus *Rhodococcus*

- the genus *Streptomyces*;

Yeasts such as

- the genus *Saccharomyces*
- the genus *Kluyveromyces*
- the genus *Schizosaccharomyces*
- the genus *Zygosaccharomyces*
- the genus *Yarrowia*
- the genus *Trichosporon*
- the genus *Rhodosporidium*
- the genus *Pichia*
- the genus *Candida*; and

Fungi such as

- the genus *Neurospora*
- the genus *Aspergillus*
- the genus *Cephalosporium*
- the genus *Trichoderma*.

Procedures for preparing a transformant and constructing a recombinant vector suitable for a host can be carried out by employing techniques commonly used in the fields of molecular biology, bioengineering, and genetic engineering (for example, see Sambrook *et al.*, "Molecular Cloning" , Cold Spring Harbor Laboratories (2001)). To express in a microorganism a gene that encodes a carbonyl reductase of the present invention, which utilizes NADPH as an electron donor, it is necessary to introduce the DNA into a plasmid vector or phage vector that is stable in that microorganism, and to transcribe and translate the genetic information.

To achieve this, a promoter, a unit for regulating transcription and translation, is placed upstream of the 5' end of the DNA of the present invention, and preferably, a terminator is placed downstream of the 3' end of the DNA. The promoter and terminator should be functional in the microorganism to be used as host. Available vectors, promoters, and terminators for the above-mentioned various microorganisms are described in detail in "Fundamental Course in Microbiology (8): Genetic Engineering", Kyoritsu Shuppan, specifically for yeasts, in "Adv. Biochem. Eng. 43, 75-102 (1990)" and "Yeast 8, 423-488 (1992)".

For example, for the genus *Escherichia*, and in particular for *Escherichia coli*, available plasmids include the pBR series and pUC series plasmids; available promoters include promoters derived from lac (derived from β-galactosidase gene), trp (derived from the tryptophan operon), tac and trc (which are chimeras of lac and trp), and PL and PR of λ phage. Available terminators are derived from trpA, phages, rrnB ribosomal RNA, etc. Of these, vector pSE420D (described in JP-A 2000-189170), which is produced by modifying a part of multicloning site of commercially available pSE420 (Invitrogen), can be suitably utilized.

For the genus *Bacillus*, available vectors are pUB110 series and pC194 series plasmids, and can be integrated into a host chromosome. Available promoters and terminators are derived from apr (alkaline protease), npr (neutral protease), amy (α-amylase), etc.

For the genus *Pseudomonas*, there are host-vector systems developed for *Pseudomonas putida* and *Pseudomonas cepacia*. A broad-host-range vector, pKT240, (comprising RSF1010-derived genes required for autonomous replication) based on TOL plasmid, which is involved in decomposition of toluene compounds, is available. A promoter and a terminator derived from the lipase gene (JP-A Hei 5-284973) are also available.

For the genus *Brevibacterium*, and in particular for *Brevibacterium lactofermentum*, available plasmid vectors include pAJ43 (Gene 39, 281-286 (1985)). Promoters and terminators used for *Escherichia coli* can be utilized for *Brevibacterium* without any modification.

For the genus *Corynebacterium*, and in particular for *Corynebacterium glutamicum*, plasmid vectors such as pCS11 (JP-A Sho 57-183799) and pCB101 (Mol. Gen. Genet. 196, 175 (1984)) are available.

For the genus *Streptococcus*, plasmid vectors such as pHV1301 (FEMS Microbiol. Lett. 26, 239 (1985)) and pGK1 (Appl. Environ. Microbiol. 50, 94 (1985)) can be used.

For the genus *Lactobacillus*, plasmid vectors such as pAMβ1 (J. Bacteriol. 137, 614 (1979)), which was developed for the genus *Streptococcus*, can be utilized. Promoters that are used for *Escherichia coli* can also be used.

For the genus *Rhodococcus*, plasmid vectors isolated from *Rhodococcus rhodochrous* are available (J. Gen. Microbiol. 138, 1003 (1992)).

For the genus *Streptomyces*, plasmids can be constructed in accordance with the method described in "Genetic Manipulation of *Streptomyces*: A Laboratory Manual" (Hopwood *et al*, Cold Spring Harbor Laboratories (1985)). In particular, for *Streptomyces lividans*, pIJ486 (Mol. Gen. Genet. 203, 468-478, 1986), pKC1064 (Gene 103, 97-99 (1991)), and pUWL-KS (Gene 165, 149-150 (1995)) can be used. The same plasmids can also be utilized for *Streptomyces virginiae* (Actinomycetol. 11, 46-53 (1997)).

For the genus *Saccharomyces*, and in particular for *Saccharomyces cerevisiae*, YRp series, YEp series, YCp series, and YIp series plasmids are available. In addition, integration vectors (see EP 537456, and such), which are integrated into a chromosome via homologous recombination with multicopy-ribosomal genes, allow the introduction of a gene of interest in multicopy, and the gene incorporated can be stably maintained in the microorganism. Thus, this type of vector is highly useful. Available promoters and terminators are derived from genes encoding alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), acid phosphatase (PHO), β-galactosidase (GAL), phosphoglycerate kinase (PGK), enolase (ENO), etc.

For the genus *Kluyveromyces*, in particular, for *Kluyveromyces lactis*, available plasmids are those such as 2-μm plasmids derived from *Saccharomyces cerevisiae*, pKD1 series plasmids (J. Bacteriol. 145, 382-390 (1981)), plasmids derived from pGKl1 and involved in killer activity, KARS (*Kluyveromyces* autonomous replication sequence) series plasmids, and plasmids capable of being integrated into a chromosome via homologous recombination with ribosomal DNA (see, EP 537456, etc.). Promoters and terminators derived from ADH, PGK, and so on are also available.

For the genus *Schizosaccharomyces*, it is possible to use plasmid vectors comprising autonomous replication sequence (ARS) derived from *Schizosaccharomyces pombe* and auxotrophy-complementing selectable markers derived from *Saccharomyces cerevisiae* (Mol. Cell. Biol. 6,

80 (1986)). Promoters such as ADH promoter derived from *Schizosaccharomyces pombe* can also be used (EMBO J. 6, 729 (1987)). In particular, pAUR224 is commercially available from TaKaRa Shuzo Co., Ltd.

For the genus *Zygosaccharomyces*, plasmid vectors originating from those such as pSB3 (Nucleic Acids Res. 13, 4267 (1985)) derived from *Zygosaccharomyces rouxii* are available. In addition, it is possible to use promoters such as PHO5 promoter derived from *Saccharomyces cerevisiae* and GAP-Zr (Glyceraldehyde-3-phosphate dehydrogenase) promoter (Agri. Biol. Chem. 54, 2521 (1990)) derived from *Zygosaccharomyces rouxii*.

For the genus *Pichia*, a host vector system has been developed for *Pichia angusta* (previously called *Hansenula polymorpha*). Although *Pichia angusta*-derived autonomous replication sequences (HARS1 and HARS2) are available as vectors, they are rather unstable, and thus multicopy chromosomal integration is effective (Yeast 7, 431-443 (1991)). In addition, methanol-induced promotors of alcohol oxidase (AOX) and formate dehydrogenase (FDH) and the like are available. Furthermore, host-vector systems originating from *Pichia*-derived autonomous replication sequences (PARS1, PARS2) have been developed (Mol. Cell. Biol. 5, 3376 (1985)). A highly efficient promoter, such as AOX promoter, which is inducible by high-cell-density-culture and methanol can also be employed (Nucleic Acids Res. 15, 3859 (1987)).

In the genus *Candida*, host-vector systems have been developed for *Candida maltosa*, *Candida albicans*, *Candida tropicalis*, *Candida utilis*, etc. An ARS originating from *Candida maltosa* has been cloned (Agri. Biol. Chem. 51, 51, 1587 (1987)), and a vector using this sequence has been developed for *Candida maltosa*. Furthermore, a chromosome-integration vector with a highly efficient promoter has been developed for *Candida utilis* (JP-A Hei 08-173170).

Of fungi, *Aspergillus niger* and *Aspergillus oryzae* of the genus *Aspergillus* have been extensively studied, and thus plasmid vectors and chromosome-integration vectors are available, as well as promoters derived from an extracellular protease gene and amylase gene (Trends in Biotechnology 7, 283-287 (1989)).

In the genus *Trichoderma*, host-vector systems have been developed

for *Trichoderma reesei*, and promoters such as those derived from extracellular cellulase genes are available (Biotechnology 7, 596-603 (1989)).

Various host-vector systems have also been developed for plants and animals. In particular, systems include those of insects such as silkworm (Nature 315, 592-594 (1985)), and plants such as rapeseed, maize, and potato. These systems are preferably used to express a large amount of foreign protein.

The microorganisms with the ability to produce carbonyl reductase used in this invention comprise all strains, mutants, and variants with the ability to produce carbonyl reductase, as well as transformants constructed by genetic engineering and with the ability to produce the enzyme of the present invention.

The present invention relates to methods for producing an optically active alcohol, in particular, for producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol by reducing ketones utilizing an aforementioned carbonyl reductase, and to uses of this alcohol. A preferred embodiment of the present invention provides a method for producing an enzyme of the present invention, where the method comprises the step of culturing a microorganism of the genus *Torulaspora* which produces a carbonyl reductase of the present invention. Furthermore, the present invention provides a method for producing a carbonyl reductase of the present invention, which comprises the step of culturing the aforementioned transformant of the present invention. An optically active alcohol can be produced using the desired enzyme reaction, performed by contacting a reaction solution with an enzyme molecule, a treated enzyme molecule, a culture comprising an enzyme molecule, or a transformant such as a microorganism that produces an enzyme. The specific mode by which an enzyme is contacted with a reaction solution is not limited to these particular examples. Examples of a microorganism used in the aforementioned methods preferably include *Torulaspora delbrueckii*.

A preferred embodiment of a method for producing an optically active alcohol of the present invention provides a method for producing an optically active alcohol, which comprises reacting a ketone with a carbonyl reductase of the present invention, a protein of the present

invention, a microorganism which produces the enzyme or the protein, the treated microorganism, a transformant of the present invention, or a carbonyl reducing agent of the present invention. In addition, one example of the methods of the present invention is a method for producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol, which comprises reacting 3,4-dimethoxyphenylacetone with a carbonyl reductase of the present invention, a protein of the present invention, a microorganism which produces the enzyme or the protein, the treated microorganism, a transformant of the present invention, or a carbonyl reducing agent of the present invention.

More specifically, examples of the treated microorganism comprising carbonyl reductase in the present invention comprise a microorganism in which the permeability of a cell membrane has been changed by treatment with a surfactant or an organic solvent such as toluene, a cell-free extract obtained by disrupting cells by treatment with glass beads or an enzyme, and a partially purified extract.

As a ketone in a method for producing an optically active alcohol of the present invention, 1-acetoxy-2-propanone, ethyl acetoacetate, methyl acetoacetate, ethyl 4-chloroacetoacetate, methyl 4-chloroacetoacetate, 2-chloro-1-(3'-chlorophenyl)ethanone, or 3,4-dimethoxyphenylacetone can be preferably used, and (S)-1-acetoxy-2-propanol, ethyl (S)-3-hydroxybutanoate, methyl (S)-3-hydroxybutanoate, ethyl (R)-4-chloro-3-hydroxybutanoate, methyl (R)-4-chloro-3-hydroxybutanoate, (R)-2-chloro-1-(3'-chlorophenyl)ethanol, and (S)-1-(3,4-dimetoxyphenyl)-2-propanol can be produced.

A preferred embodiment of the present invention provides a method for producing (S)-1-(3,4-dimethoxyphenyl)-2-propanol, which comprises reacting 3,4-dimethoxyphenylacetone with a carbonyl reductase of the present invention, a protein comprising the carbonyl-reducing activity, a microorganism which produces the enzyme or the protein, or the treated microorganism.

NADPH regeneration using the $NADP^+$ produced from NADPH in the aforementioned reduction reaction can be performed using the $NADP^+$-reducing ability of microorganisms (glycolysis system, C1

assimilation pathway of methylotrophs, etc.). $NADP^+$-reducing ability can be enhanced by adding glucose or ethanol to a reaction system. Alternatively, reducing ability can also be enhanced by adding to the reaction system a microorganism with the ability to produce NADPH from $NADP^+$, or that treated microorganism, or an enzyme. For example, NADPH regeneration can be performed by using a microorganism comprising glucose dehydrogenase, alcohol dehydrogenase, formate dehydrogenase, amino acid dehydogenase, or organic acid dehydrogenase (such as malate dehydrogenase), the treated microorganism, or a partially purified or purified enzyme. These components, which constitute a necessary reaction for regenerating NADPH, can be contacted by addition to a reaction system for producing an optically active alcohol of the present invention, by adding immobilized components thereto, or by using a membrane that can exchange NADPH.

The present invention also relates to methods for producing a protein comprising the carbonyl-reducing activity of the present invention, which include the step of culturing a transformant transformed with a recombinant vector comprising a polynucleotide of the present invention. In some cases in the present methods, when a live cell of a microorganism, transformed with a recombinant vector comprising a polynucleotide of the present invention, is utilized in a method for producing the aforementioned optically active alcohol, an additional reaction system for regenerating NADPH may be unnecessary. Specifically, by using a microorganism with a high NADPH regenerating activity, the reduction reaction using a transformant can be efficiently performed without the addition of an NADPH-regenerating enzyme. Furthermore, the host can be introduced both with a DNA encoding an NADPH-dependent carbonyl reductase of the present invention, and a gene useful in regenerating NADPH (a coenzyme regenerating dehydrogenase gene), for example that of a glucose dehydrogenase, alcohol dehydrogenase, formate dehydrogenase, amino acid dehydrogenase, or organic acid dehydrogenase (such as malate dehydrogenase). This will result in more efficient expression of the NADPH-regenerating enzyme and the NADPH-dependent carbonyl reductase, and a more efficient reduction reaction. When introducing two or more of these genes into a host, in order to avoid incompatibility, methods

such as the following can be used: a method for transforming the host with multiple recombinant vectors into which genes have been separately introduced and where the vectors have different replication origins; a method in which the two or more genes are introduced into a single vector; or a method for introducing a number of or one of the genes into chromosomes.

When multiple genes are introduced into a single vector, each gene can be ligated to a region involved in the regulation of expression, such as a promotor or terminater. Multiple genes can also be expressed as an operon comprising multiple cistrons, such as the lactose operon.

As an NADPH-regenerating enzyme, for example, a glucose dehydrogenase derived from *Bacillus subtilis* or *Thermoplasma acidophilum* can be utilized. Specifically, preferably utilized recombinant vectors include pSG-TDR1, pSG-YDR1, pSG-YGP7, and pSG-YGD9 vectors into which a carbonyl reductase gene and a glucose dehydrogenase gene derived from *Bacillus subtilis* have been introduced.

A reduction reaction using an enzyme of the present invention can be performed in water, in a water-insoluble organic solvent such as ethyl acetate, butyl acetate, toluene, chloroform, n-hexane, methyl isobutyl ketone, and methyl tertiary butyl ester; in a two-phase system with an aqueous medium, or in a mixture system with a water-soluble organic solvent such as methanol, ethanol, isopropyl alcohol, acetonitrile, acetone, and dimethyl sulfoxide. The reaction in the present invention may be performed by utilizing an immobilized enzyme or a membrane reactor.

The reaction of the present invention can be performed at a temperature range of 4°C to 60°C, preferably at 15°C to 37°C; at a pH of 3 to 11, preferably pH 5 to 9; and at a substrate concentration of 0.01% to 50%, preferably 0.1% to 20%, more preferably 0.1% to 10%. If necessary, coenzyme $NADP^+$ or NADPH may be added to the reaction system at 0.001 mM to 100 mM, more preferably at 0.01 mM to 10 mM. Although a substrate may be added at once at the beginning of the reaction, it is preferable to add it continuously or discontinuously, such that the substrate concentration in the reaction solution does not become too high.

When regenerating NADPH, for example, glucose (when using glucose

dehydrogenase) or ethanol/ isopropanol (when using alcohol dehydrogenase) is added to the reaction system. These compounds may be added at a molar ratio relative to a substrate ketone of 0.1- to 20-fold, preferably in excess at 1- to 5-fold. On the other hand, a NADPH-regenerating enzyme such as glucose dehydrogenase or alcohol dehydrogenase can be added at approximately 0.1- to 100-fold, and preferably at 0.5- to 20-fold of the enzyme activity compared with the NADPH-dependent carbonyl reductase of the present invention.

Purification of an optically active alcohol produced by reduction of a ketone in the present invention can be performed by appropriately combining the centrifugation of cells and proteins, separation by membrane treatment, solvent extraction, distillation, etc.

For example, (S)-1-(3,4-dimethoxyphenyl)-2-propanol can be obtained as an optically active alcohol by centrifuging a reaction solution comprising microorganism cells to remove the cells, extracting with ethyl acetate, butyl acetate, toluene, hexane, benzene, methyl isobutyl ketone, methyl tertiary butyl ether, or butanol, and then performing vacuum concentration. The purity of the reaction product can be further increased by using silica gel column chromatography, etc.

NADPH-dependent carbonyl reductases useful for producing an optically active alcohol has been provided. By utilizing the present enzymes, methods for efficiently producing high optical purity (S)-1-(3,4-dimethoxyphenyl)-2-propanol from 3,4-dimethoxyphenylacetone have been provided.

The words "a", "an", and "the" as used herein mean "at least one" unless otherwise specifically indicated. Furthermore, all patents, published patent applications, and publications cited herein are incorporated by reference in their entirety.

Herein, the present invention will be specifically described below using Examples. However, it is not to be construed as being limited thereto.

EXAMPLE 1

Purification of a carbonyl reductase

Cells for enzyme purification were prepared by culturing *Torulaspora delprueckii* JCM 5921 strain in 1.2 L of YM medium (glucose

20 g/L, yeast extract 3 g/l, malt extract 3 g/L, peptone 5 g/L, pH 6.0), followed by centrifugation. The resulting wet cells were suspended in a solution containing 50 mM Tris-HCl buffer (pH 8.5), 0.02% 2-mercaptoethanol, and 2 mM phenylmethanesulfonyl fluoride (PMSF), and homogenized with a bead beater (Biospec). The cell residue was then removed by centrifugation to obtain a cell-free extract. Protamine sulfate was added to the cell-free extract, which was then centrifuged to remove nucleic acids and obtain the supernatant. Ammonium sulfate was then added to the supernatant to 30% saturation, and this was added to Phenyl-Sepharose™ HP (2.6cm x 10cm) equilibrated with a standard buffer (10 mM Tris-HCl buffer (pH 8.5), 0.01% 2-mercaptoethanol, and 10% glycerol) comprising 30% ammonium sulfate. The present enzyme was then eluted using ammonium sulfate over a concentration gradient of 30% to 0%. NADPH-dependent 3,4-dimethoxyphenylacetone-reducing activity was observed in the gradient eluted fractions, and the eluted peak part was collected and then concentrated by ultrafiltration.

The concentrated enzyme solution was dialyzed against a standard buffer, added to MonoQ (0.5cm x 5cm) equilibrated with the same buffer, and subjected to elution with sodium chloride over a gradient concentration of 0 M to 0.5 M. The eluted active fraction was collected, and subjected to ultrafiltration to obtain the concentrated enzyme solution.

The concentrated enzyme solution was dialyzed against 5 mM potassium phosphate buffer (pH 8.0) comprising 0.01% 2-mercaptoethanol and 10% glycerol, added to a hydroxyapatite column (0.5cm x 10cm) equilibrated with the same buffer, and then subjected to gradient elution with potassium phosphate buffer (pH 8.0) over 5 mM to 350 mM. The eluted active fraction comprising the highest specific activity was analyzed using SDS-PAGE. As a result, a single band consisting of only the present enzyme was obtained (Fig. 1).

The specific activity of the purified enzyme was 196 mU/mg. A summary of the purification is shown in Table 1.

Table 1

Step	Protein (mg)	Enzyme activity (U)	Specific activity (mU/mg)
Cell-free extract	15,000	-	-
Nucleic acid removal	6,580	24.1	3.66
Butyl-Toyopearl	377	7.00	18.6
MonoQ	31.4	3.75	119
Hydroxyapatite	0.245	0.048	196

EXAMPLE 2

Measurement of molecular weight of the carbonyl reductase

The molecular weight of a subunit of the enzyme obtained in Example 1 was about 38,000, as determined using SDS-PAGE. The molecular weight was also measured separately using a Superdex G200 gel filtration column, and was again found to be about 38,000. Therefore, the present enzyme was presumed to be a monomer.

EXAMPLE 3

Optimal pH

By changing the pH using potassium phosphate buffer, sodium acetate buffer, and Britton-Robinson buffer, the 3,4-dimethoxyphenylacetone-reducing activity of the enzyme obtained in Example 1 was investigated. Activity at each pH was expressed as a relative activity, where the maximum activity was regarded as 100 (Fig. 2). Optimal pH (showing 80% or more relative activity) was 5.5 to 6.5.

EXAMPLE 4

Optimal temperature

Of the standard reaction conditions, only the temperature was changed, and the 3,4-dimethoxyphenylacetone-reducing activity of the enzyme obtained in Example 1 was then measured. Activity at each temperature was expressed as a relative activity, where the maximum activity was regarded as 100(Fig. 3). Optimal temperature (showing 80% or more relative activity) was 50°C to 55°C.

EXAMPLE 5

Substrate specificity

The enzyme obtained in Example 1 was reacted with various ketones, ketoesters, and so on. The activities of the reduction reactions were expressed as relative activities, where the reduction of 3,4-dimethoxyphenylacetone was regarded as 100 (Table 2) (substrate specificity of carbonyl reductase). The activity of dehydrogenating 1-(3,4-dimethoxyphenyl)-2-propanol was measured as follows: A reaction was performed at 30°C in a reaction solution comprising 50 mM Tris-HCl buffer (pH 8.5), 2.5 mM $NADP^+$, 5 mM 1-(3,4-dimethoxyphenyl)-2-propanol, and the enzyme. An increase in absorbance at 340 nm, accompanying NADPH production, was measured. 1U was defined as the amount of enzyme catalyzing the production of 1 μmol NADPH per minute. In addition, the activity of reducing NADH-dependent 3,4-dimethoxyphenylacetone was measured as follows: A reaction was performed at 30°C in a reaction solution comprising 50 mM potassium phosphate buffer (pH 6.5), 0.2 mM NADH, 5 mM 3,4-dimethoxyphenylacetone, and the enzyme, and decrease in absorbance at 340 nm, accompanying decrease in NADH, was measured. 1U was defined as the amount of enzyme catalyzing a decrease of 1 μmol NADH per minute.

Table 2

Substrate	Concentration (mM)	Coenzyme	Relative activity (%)
3,4-Dimethoxyphenylacetone	5	NADPH	100
3,4-Dimethoxyphenylacetone	5	NADH	0.0
(S)-1-(3,4-dimethoxyphenyl)-2-propanol	5	$NADP^+$	0.0
(R)-1-(3,4-dimethoxyphenyl)-2-propanol	5	$NADP^+$	0.0
Ethyl acetoacetate	20	NADPH	3170
Methyl acetoacetate	20	NADPH	3670
Ethyl 4-chloroacetoacetate	20	NADPH	6780
Methyl 4-chloroacetoacetate	20	NADPH	8040
2-Chloro-1-(3'-chlorophenyl)ethanone	2	NADPH	922
2,3-Butanedione	20	NADPH	1480
2,4-Pentanedione	20	NADPH	422
Acetophenone	20	NADPH	256
1-Acetoxy-2-propanone	20	NADPH	24700
2-Acetoxycyclopentanone	20	NADPH	567
4'-Methoxypropiophenone	20	NADPH	283
Benzylacetone	20	NADPH	739
Phenoxy-2-propanone	20	NADPH	939
2-Acetoxy-3-butanone	20	NADPH	589
Methyl pyruvate	20	NADPH	1320
Camphorquinone	1	NADPH	644
2,3-Pentanedione	20	NADPH	2590
Methoxyacetone	20	NADPH	117

EXAMPLE 6

Synthesis of (S)-1-(3,4-dimethoxyphenyl)-2-propanol using the carbonyl reductase

A reaction was performed overnight at 25°C in 1 mL of a reaction solution comprising 200 mM potassium phosphate buffer (pH 6.5), 1 mM $NADP^+$, 2U glucose dehydroganase (Wako Pure Chemical Industries, Ltd.), 250 mM glucose, 0.25U carbonyl reductase, and 50 mM 3,4-dimethoxyphenylacetone. The optical purity of the produced (S)-1-(3,4-dimethoxyphenyl)-2-propanol was measured as follows: One mL of ethyl acetate was added to 0.5 mL of the reaction solution to extract (S)-1-(3,4-dimethoxyphenyl)-2-propanol, and the extraction

solvent was desolvated. 0.5 mL of a dissolving solution (n-hexane: isopropanol = 4:1) was then added to dissolve the extract, which was then analyzed by liquid chromatography using an optical resolution column. CHIRALCEL OF (4.6 mmx 25 cm; Daicel Chemical Industries, Ltd.) was used as the optical resolution column. Chromatography was performed with a wavelength of 220 nm, a flow rate of 1.0 mL/min, and at 40°C using an eluting solution of n-hexane: isopropanol = 4:1. The (S)-1-(3,4-dimethoxyphenyl)-2-propanol produced by the present invention had a purity of 99%ee or more.

In addition, the (S)-1-(3,4-dimethoxyphenyl)-2-propanol thus-produced was quantified using gas chromatography, and a yield relative to the raw starting material, 3,4-dimethoxyphenylacetone, was obtained. Specifically, the analysis was performed at a column temperature of 210°C, using Thermon 3000(10%)-Chromosorb W (AW-DMCS, Mesh 60-80; 3.2 mmx 210 cm) with a hydrogen flame ionization detector (FID). The resulting reaction yield was about 95%.

Example 7

Partial amino acid sequence of the carbonyl reductase

The N-terminal amino acid sequence of the enzyme obtained in Example 1 was analyzed using a protein sequencer. The amino acid sequence is shown in SEQ ID NO: 3. In addition, a gel fragment comprising carbonyl reductase was excised from an SDS-PAGE gel, washed twice, and subjected to overnight in-gel digestion at 35°C, using a lysyl endopeptidase. The digested peptide was separated and collected by gradient elution of acetonitrile in 0.1% trifluoroacetic acid using reverse phase HPLC (TSK gel ODS-80-Ts, 2.0 mmx 250 mm; Tosoh Corporation).

The obtained peptide peak was named lep_41, and its amino acid sequence was analyzed using a protein sequencer (Hewlett Packard G1005A Protein Sequence System). The amino acid sequence of lep_41 is shown as SEQ ID NO: 4.

EXAMPLE 8

Purification of chromosomal DNA from *Torulaspora delbrueckii*

Cells were prepared by culturing *Torulaspora delbrueckii* JCM

5921 strain on YM medium. Chromosomal DNA was purified from these cells using the method described in Meth. Cell Biol. 22, 39-44 (1975).

EXAMPLE 9

Cloning of the core region of the carbonyl reductase gene

Based on the amino acid sequences of the N-terminus and lep_41, sense and antisense primers were synthesized. Each nucleotide sequence is shown in SEQ ID NOs: 5 (TdCr-N1) and 6 (TdCR-41).

Using 50 μL of a reaction solution comprising 50 pmol of both primers TdCR-N1 and TdCR-41, 10 nmol of dNTP, 50 ng of *Torulaspora delbrueckii*-derived chromosomal DNA, Ex-Taq buffer (TAKARA SHUZO CO., Ltd.), and 2U of Ex-Taq (TAKARA SHUZO CO., Ltd.), 30 cycles of denaturation (94°C, 30 seconds), annealing (51°C, 30 seconds), and elongation (70°C, 20 seconds) were performed using GeneAmp® PCR System 2400 (Applied Biosystems).

A part of the PCR reaction solution was analyzed by agarose gel electrophoresis. As a result, a band that seemed specific could be detected at around 330 bp. The resulting DNA fragment was extracted using phenol/chloroform, precipitated with ethanol and then collected and digested with restriction enzyme EcoRI. The digested DNA was subjected to agarose gel electrophoresis, and the desired band was excised to be purified by Sephaglas Band Prep Kit (Amersham Biosciences).

The resulting DNA fragment was ligated using EcoRI-digested pUC18 (TAKARA SHUZO Co., Ltd.) and a Takara Ligation Kit, and then used to transform the *Escherichia coli* JM109 strain.

The transformants were grown on plates of LB medium (1% Bacto-triptone, 0.5% Bacto-yeast extract, and 1% sodium chloride; hereinafter, abbreviated as LB medium) comprising ampicillin (50 μg/mL). A number of white colonies were selected by the Blue/White selection method, cultured in liquid LB medium comprising ampicillin, and a plasmid was purified by FlexiPrep (Amersham Biosciences) to obtain pTDR.

Using the purified plasmid, the nucleotide sequence of the inserted DNA was analyzed. PCR was performed using BigDye™ Terminator Cycle Sequencing FS ready Reaction Kit (Applied Biosystems), and the

PCR product was analyzed using a DNA sequencer ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). The determined nucleotide sequence of the core region is shown as SEQ ID NO: 7.

EXAMPLE 10

Analysis of the nucleotide sequence at the periphery of the core region of the carbonyl reductase gene

Torulaspor delbruckii-derived chromosomal DNA was digested with restriction enzyme BamHI, and each fragment was cyclized at 16°C overnight by a self-ligation reaction using T4 ligase. Next, using 50 L of a reaction solution comprising 100 pmol of each of the primers TdCR-59 (SEQ ID NO: 8) and TdCR-234 (SEQ ID NO: 9), as well as 25 ng of the cyclized DNA, Ex-Taq buffer (TAKARA SHUZO Co. Ltd.), and 2 U of Ex-Taq (TAKARA SHUZO Co. Ltd.), 30 cycles of denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds), and elongation (72°C, seven minutes) were performed using GeneAmp® PCR System 2400 (Applied Biosystems). A part of the PCR reaction solution was analyzed by agarose gel electrophoresis, and a DNA fragment of about 2000 bp was detected. This DNA fragment was purified using a Sephaglas BandPrep Kit (Amersham Biosciences), and a nucleotide sequence was analyzed by the primer walking method. As a result, the ORF sequence of a carbonyl reductase gene was determined. The DNA sequence thus-determined is shown in SEQ ID NO: 1, and the predicted amino acid sequence is shown in SEQ ID NO: 2. The ORF search was performed using Genetyx-win software (Genetix Corporation).

EXAMPLE 11

Construction of plasmid pSE-TDX comprising a part of the carbonyl reductase gene TdCR1

The primers Td-ATG1 (SEQ ID NO: 10) and Td-XbaR (SEQ ID NO: 11) were synthesized to clone from the 5'-terminus to an XbaI site of the ORF of the carbonyl reductase gene.

Using 50 μL of a reaction solution comprising 50 pmol of each of the primers Td-ATG1 and Td-XbaR, as well as 10 nmol of dNTP, 50 ng of a chromosomal DNA derived from *Torulaspora delbruectii*, Pfu Turbo DNA polymerase buffer (Stratagene), and 3.75 U of Pfu Turbo

DNA polymerase (Stratagene), 30 cycles of denaturation (95°C, two minutes 30 seconds), annealing (55°C, one minute), and elongation (72°C, one minute) were performed using a GeneAmp® PCR System 2400 (Applied Biosystems). The resulting PCR product was designated as Td-PCR1.

The resulting PCR product was collected by extraction with phenol-chloroform and ethanol precipitation. The Td-PCR1 was digested with two restriction enzymes BspHI and XbaI, and subjected to agarose gel electrophoresis. The desired band was excised and then purified by Sephaglas BandPrep Kit (Amersham Biosciences).

A Takara Ligation Kit was used to ligate the restriction enzyme-digested Td-PCR1 with the vector pSE420D (JP-A 2000-189170), which had been digested with two restriction enzymes, NcoI and XbaI. *Escherichia coli* JM109 strain was transformed with the ligated plasmid. The transformants were grown on LB medium comprising ampicillin, and the nucleotide sequence of the inserted fragment was analyzed. The obtained plasmid was designated as pSE-TDX. The process of constructing this plasmid is shown in Fig. 4.

EXAMPLE 12

Construction of plasmid pUC-TDX comprising a part of the carbonyl reductase gene TdCR1

Primers Td-XbaF (SEQ ID NO: 12) and Td-TAA1 (SEQ ID NO: 13) were synthesized for cloning from the XbaI site to the 3'-terminus of the ORF of the carbonyl reductase gene.

Using 50 μL of a reaction solution comprising 50 pmol of each of the primers Td-XbaF and Td-TAA1, as well as 10 nmol of dNTP, 50 ng of *Torulaspora delbruectii*-derived chromosomal DNA, Pfu Turbo DNA polymerase buffer (Stratagene), and 3.75 U of Pfu Turbo DNA polymerase (Stratagene), 30 cycles of denaturation (95°C, two minutes 30 seconds), annealing (55°C, one minute), and elongation (72°C, one minute) were performed using a GeneAmp® PCR System 2400 (Applied Biosystems). The resulting PCR product was designated as Td-PCR2.

The resulting PCR product was collected by extraction using phenol-chloroform and ethanol precipitation. The Td-PCR2 was digested with XbaI, and subjected to agarose gel electrophoresis. The desired band was excised and then purified by Sephaglas BandPrep

Kit (Amersham Biosciences).

A Takara Ligation Kit was used to ligate the restriction enzyme-digested Td-PCR2 with the vector pUC18, which had been digested with XbaI. *Escherichia coli* JM109 strain was transformed with the ligated plasmid. The transformants were grown on LB medium comprising ampicillin, and the nucleotide sequence of the inserted fragment was analyzed. The obtained plasmid was designated as pUC-TDX. The process of constructing this plasmid is shown in Fig. 5.

EXAMPLE 13

Construction of plasmid pSG-TDX comprising a part of the carbonyl reductase gene TdCR1

Td-PCR1, the PCR product obtained by the method of Example 11, was collected by extraction with phenol-chloroform and ethanol precipitation. The resulting DNA was digested with two restriction enzymes, BspHI and XbaI, and subjected to agarose gel electrophoresis. The desired band was excised and then purified using a Sephaglas BandPrep Kit (Amersham Biosciences).

A Takara Ligation Kit was used to ligate the restriction enzyme-digested Td-PCR1 with the vector pSE-BSG1 (JP-A 2000-189170), which comprises a *Bacillus subtilis*- derived glucose dehydrogenase gene and had been digested with restriction enzymes NcoI and XbaI. *Escherichia coli* JM109 strain was transformed with the ligated plasmid. The transformants were grown on LB medium comprising ampicillin, and the nucleotide sequence of the inserted fragment was analyzed. The obtained plasmid was designated as pSG-TDX. The process of constructing this plasmid is shown in Fig. 6.

EXAMPLE 14

Construction of plasmid pSE-TDR1 expressing the carbonyl reductase gene TdCR1

The plasmid pUC-TDX obtained in Example 12 was digested using restriction enzyme XbaI, ethanol-precipitated, and then subjected to agarose electrophoresis. A band of about 0.8 kb, comprising part of the TdCR1 gene, was excised. This band was purified by Sephaglas Band Prep (Amersham Biosciences) and collected. A TaKaRa Ligation

Kit was used to ligate the resulting DNA fragment with a plasmid pSE-TDX, obtained by digestion with the same restriction enzyme, treatment with alkaline phosphatase, phenol extraction, phenol/chloroform extraction, chloroform extraction, and ethanol precipitation. *Escherichia coli* JM109 strain was transformed with the ligated DNA and cultured on LB medium comprising ampicillin (50 mg/L). A plasmid was purified from the resulting transformants using a Flexi Prep kit. As a result, the plasmid pSE-TDR1, which comprises the full length TdCR1 gene and can express TdCR1, was obtained. The process for constructing this plasmid is shown in Fig. 7.

EXAMPLE 15

Construction of plasmid pSG-TDR1, which coexpresses the carbonyl reductase gene TdCR1 and a *Bacillus subtilis*-derived glucose dehydrogenase gene

The plasmid pUC-TDX obtained in Example 12 was digested with restriction enzyme XbaI, ethanol-precipitated, and then subjected to agarose electrophoresis. A band of about 0.8 kb comprising a part of the TdCR1 gene was excised. This band was purified by Sephaglas Band Prep (Amersham Biosciences) and collected. A TaKaRa Ligation Kit was used to ligate the resulting DNA fragment with plasmid pSG-TDX, obtained by digestion with the same restriction enzyme, treatment with alkaline phosphatase, phenol extraction, phenol/chloroform extraction, chloroform extraction, and ethanol precipitation. *Escherichia coli* JM109 strain was transformed with the ligated DNA and cultured on LB medium comprising ampicillin (50 mg/L). A plasmid was purified with Flexi Prep kit from the resulting transformants. As a result, plasmid pSG-TDR1, which can coexpress the glucose dehydrogenase gene and TdCR1, was obtained. The process for constructing this plasmid is shown in Fig. 8.

EXAMPLE 16

Confirmation of carbonyl reductase activity

Escherichia coli JM109 strain transformed with plasmid pSE-TDR1, which expresses carbonyl reductase, and plasmid pSG-TDR1, which coexpresses carbonyl reductase and *Bacillus subtilis*-derived glucose

dehydrogenase, was cultured overnight at 30°C in liquid LB medium containing ampicillin. 0.1 mM IPTG was added, and the strain was further cultured for four hours.

Cells were collected by centrifugation, and then suspended in 50 mM potassium phosphate buffer (pH 6.5) comprising 0.5 M NaCl, 0.02% 2-mercaptoethanol, 2 mM PMSF, and 10% glycerin. Cells were then disrupted by sonication, and centrifuged. The obtained supernatant was used as a cell-free extract. The 3,4-dimethoxyphenylacetone-reducing activity and glucose-dehydrogenating activity of the cell-free extract were measured. The glucose-dehydrogenating activity was measured as follows: Measurements were performed at 30°C in a reaction solution comprising the enzyme and 100 mM potassium phosphate buffer (pH 6.5), 2.5 mM NAD^+, 100 mM glucose. 1 U was defined as the amount of enzyme to catalyze production of 1 mol of NADH per minute under the aforementioned reaction conditions.

Table 3 shows the results of measuring the 3,4-dimethoxyphenylacetone-reducing activity and glucose dehydrogenase activity using each cell-free extract. In all cases, 3,4-dimethoxyphenylacetone-reducing activity was confirmed.

Table 3

Plasmid	3,4-Dimethoxyphenylacetone reducing activity (mU/mg protein)	Glucose dehydrogenase activity (U/mg protein)
None	0	0
pSE-TDR1	61.6	0
pSG-TDR1	57.0	4.07

EXAMPLE 17

Purification of chromosomal DNA from *Saccharomyces cerevisiae*

Saccharomyces cerevisiae X2180-1B (Yeast Genetic Stock Center) was cultured on YM medium to prepare cells. Purification of chromosomal DNA from these cells was performed by the method described in Meth. Cell Biol. 22, 39-44 (1975).

EXAMPLE 18

Cloning of carbonyl reductase homolog YGL157w

PCR primers YGL1-ATG1 (SEQ ID NO: 14) and YGL1-TAA1 (SEQ ID NO: 15) were synthesized based on a DNA sequence (DDBJ Accession No. Z48618) corresponding to predicted protein YGL157w (SWISS-PROT Accession No. P53111) registered in DDBJ.

Using 50 μL of a reaction solution comprising 25 pmol of each of the primers, as well as 10 nmol of dNTP, 50 ng of *Saccharomyces cerevisiae*-derived chromosomal DNA, Pfu DNA polymerase buffer (Stratagene), and 2 U of Pfu DNA polymerase (Stratagene), PCR of 30 cycles of denaturation (95°C, 45 seconds), annealing (55°C, 30 seconds), and elongation (72°C, one minute 20 seconds) was performed with GeneAmp® PCR System 2400 (Applied Biosystems). As a result, a specific amplification product was obtained.

A TAKARA Ligation Kit was used to ligate the amplification product, which was treated with phenol and then doubly digested with restriction enzymes BspHI and XbaI, with vector pSE420D, which had been doubly digested with restriction enzymes NcoI and XbaI. *Escherichia coli* JM 109 strain was transformed with the ligated DNA, and cultured on the LB medium comprising ampicillin (50 mg/L). FlexiPrep was used to purify a plasmid from the resulting transformants.

The nucleotide sequence of the inserted plasmid DNA was analyzed and SEQ ID NO: 19 shows the resulting sequence. This nucleotide sequence was completely consistent with that registered in DDBJ. The resulting plasmid was designated as pSE-YGP7. The amino acid sequence predicted from the nucleotide sequence of SEQ ID NO: 16 is shown in SEQ ID NO: 17. The process for constructing this plasmid is shown in Fig. 9.

EXAMPLE 19

Construction of plasmid pSG-YGP7, which coexpresses carbonyl reductase homolog YGL157w and the *Bacillus subtilis*-derived glucose dehydrogenase gene

The plasmid pSE-BSG1 (JPA 2000-374593), which comprised a *Bacillus subtilis*-derived glucose dehydrogenase gene, was doubly digested with two restriction enzymes, NheI and XbaI. A Takara Ligation Kit was then used to ligate the plasmid with a DNA fragment comprising

a YGL157w gene excised from pSE-YGP7 using the same enzymes. *Escherichia coli* JM109 strain was transformed with the ligated DNA, cultured on LB medium comprising ampicillin (50 mg/L), and FlexiPrep was used to purify from the resulting transformants the plasmid pSG-YGP7, which is capable of coexpressing glucose dehydrogenase and YGL157w. The process for constructing this plasmid is shown in Fig. 10.

EXAMPLE 20

Cloning of carbonyl reductase homolog YGL039w

PCR primers YGL2-ATG2 (SEQ ID NO: 18) and YGL2-TAA2 (SEQ ID NO: 19) were synthesized based on the DNA sequence (DDBJ Accession No. Z72561) corresponding to the predicted protein YGL039w (SWISS-PROT Accession No. P53183), registered in DDBJ.

Using 50 μL of a reaction solution comprising 25 pmol of each of the primers, as well as 10 nmol of dNTP, 50 ng of *Saccharomyces cerevisiae*-derived chromosomal DNA, Pfu DNA polymerase buffer (Stratagene), and 2 U of Pfu DNA polymerase (Stratagene), PCR of 30 cycles of denaturation (95°C, 45 seconds), annealing (50°C, 30 seconds), and elongation (72°C, one minute 15 seconds) was performed with GeneAmp® PCR System 2400 (Applied Biosystems). As a result, a specific amplification product was obtained.

The amplification product was treated with phenol, doubly digested with restriction enzymes BspHI and NheI, and ligated using a TAKARA Ligation Kit with vector pSE420D, which had been doubly digested with restriction enzymes NcoI and XbaI. *Escherichia coli* JM109 strain was transformed with the ligated DNA, and cultured on LB medium comprising ampicillin (50 mg/L). FlexiPrep was used to purify a plasmid from the resulting transformants. The nucleotide sequence of the inserted plasmid DNA was analyzed, and the resulting sequence is shown in SEQ ID NO: 20. This nucleotide sequence was completely consistent with that registered in DDBJ. The resulting plasmid was designated as pSE-YGD9. The amino acid sequence predicted from the nucleotide sequence of SEQ ID NO: 20 is shown in SEQ ID NO: 21. The process for constructing this plasmid is shown in Fig. 11.

EXAMPLE 21

Construction of plasmid pSG-YGD9, which can coexpress carbonyl reductase homolog YGL039w and *Bacillus subtilis*-derived glucose dehydrogenase gene

pSE-YGD9 was doubly digested with two restriction enzymes, EcoRI and HindIII. A Takara Ligation Kit was then used to ligate the product with a DNA fragment comprising a glucose dehydrogenase gene, which was excised using the same enzymes from plasmid pSE-BSG1 (JP-A 2000-374593), which comprises the *Bacillus subtilis*-derived glucose dehydrogenase gene.

Escherichia coli JM109 strain was transformed with the ligated DNA, cultured on LB medium comprising ampicillin (50 mg/L), and FlexiPrep was used to purify from the resulting transformants the plasmid pSG-YGP9, which is capable of coexpressing glucose dehydrogenase and YGL039w. The process for constructing this plasmid is shown in Fig. 12.

EXAMPLE 22

Cloning of the carbonyl reductase homolog YDR541c

PCR primers YDR-ATG1 (SEQ ID NO: 18) and YDR -TAA1 (SEQ ID NO: 23) were synthesized based on the DNA sequence (DDBJ Accession No. Z48239) corresponding to the predicted protein YDR541c (SWISS-PROT Accession No. U43834-5), registered in DDBJ.

Using 50 μL of a reaction solution comprising 25 pmol of each of the primers, as well as 10 nmol of dNTP, 50 ng of *Saccharomyces cerevisiae*-derived chromosomal DNA, Pfu DNA polymerase buffer (Stratagene), and 2 U of Pfu DNA polymerase (Stratagene), PCR of 30 cycles of denaturation (95°C, 45 seconds), annealing (52°C, 30 seconds), and elongation (72°C, one minute 20 seconds) was performed with GeneAmp® PCR System 2400 (Applied Biosystems). As a result, a specific amplification product was obtained.

The amplification product was treated with phenol, doubly digested with restriction enzymes AflIII and XbaI, and ligated using a TAKARA Ligation Kit with vector pSE420D, which had been doubly digested with restriction enzymes NcoI and XbaI. *Escherichia coli* JM109 strain was transformed with the ligated DNA, and cultured on LB medium

comprising ampicillin (50 mg/L). FlexiPrep was used to purify a plasmid from the resulting transformants. The nucleotide sequence of the inserted plasmid DNA was analyzed, and the resulting sequence is shown in SEQ ID NO: 27. This nucleotide sequence was completely consistent with that registered in DDBJ. The resulting plasmid was designated as pSE-YDR1. The amino acid sequence predicted from the nucleotide sequence of SEQ ID NO: 24 is shown in SEQ ID NO: 25. The process for constructing this plasmid is shown in Fig. 13.

EXAMPLE 23

Construction of plasmid pSG-YGD7, which can coexpress carbonyl reductase homolog YDR541c and *Bacillus subtilis*-derived glucose dehydrogenase gene

pSE-YDR1 was doubly digested with two restriction enzymes EcoRI and HindIII. A Takara Ligation Kit was then used to ligate the product with a DNA fragment that comprised a glucose dehydrogenase gene excised using the same enzymes from plasmid pSE-BSG1 (JP-A 2000-374593), which comprised the *Bacillus subtilis*-derived glucose dehydrogenase gene.

Escherichia coli JM109 strain was transformed with the ligated DNA, cultured on LB medium comprising ampicillin (50 mg/L), and FlexiPrep was used to purify from the resulting transformants the plasmid pSG-YDR1, which is capable of coexpressing glucose dehydrogenase and YDR541c. The process for constructing this plasmid is shown in Fig. 14.

EXAMPLE 24

Confirmation of the activity of carbonyl reductase homologs YGL157w, YGL039w, and YDR541c

Escherichia coli JM109+ strains comprising pSE-YGP7, pSE-YGD9, pSE-YDR1, pSG-YGP7, pSG-YGD9, or pSG-YDR1 were cultured on LB medium comprising ampicillin, induced with 0.1 mM IPTG for four hours, and then centrifuged to collect the cells.

Each of these cell samples was suspended in a cell lysis solution (50 mM KPB pH 8.0, 1 mM EDTA, 0.02% 2-mercaptoethanol, 2 mM PMSF, 10% Glycerol). The cells were ruptured by sonication and then centrifuged to obtain the supernatant, which was used as a cell-free

extract.

3,4-dimethoxyphenylacetone-reducing activity was measured using each cell-free extract, and the results are shown in Table 4. YGL157w (pSE-YGP7 and pSG-YGP7), YGL039w (pSE-YGD9 and pSG-YGD9), and YDR541c (pSE-YDR1 and pSG-YDR1) were all confirmed to comprise 3,4-dimethoxyphenylacetone reductase activity.

Table 4

Plasmid	3,4-Dimethoxyphenylacetone reducing activity (mU/mg protein)	Glucose dehydrogenating activity (mU/mg protein)
None	0.0	0.0
pSE-YGP7	39.0	0.0
pSG-YGP7	6.85	2320
pSE-YGD9	19.1	0.0
pSG-YGD9	20.0	1340
pSE-YDR1	21.0	0.0
pSG-YDR1	30.0	30.0

EXAMPLE 25

Synthesis of (S)-1-(3,4-dimethoxyphenyl)-2-propanol by carbonyl reductase homologs YGL157w, YGL039w, and YDR541c

To prepare crude enzyme solution for enzyme reaction, the crude enzyme solution prepared in Example 18 was concentrated ten-folds using a UF membrane. This solution was reacted overnight at 25°C in 1 mL of a reaction solution comprising 200 mM potassium phosphate buffer (pH 6.5), 1 mM $NADP^+$, 2 U glucose dehydrogenase (Wako Pure Chemical Industries, Ltd.), 250 mM glucose, 0.2 U homolog enzyme, and 50 mM 3,4-dimethoxyphenylacetone. Optical purity was measured and the produced (S)-1-(3,4-dimethoxyphenyl)-2-propanol was quantified as in Example 6. As a result, YGL157w (pSE-YGP7) produced 93.7%ee (S)-1-(3,4-dimethoxyphenyl)-2-propanol at a yield of 66%, YGL039w (pSE-YGD9) produced 93.6%ee at a yield of 94%, and YDR541c (pSE-YDR1) produced 94.8%ee at a yield of 6%.